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Supplementary Materials for

Targeted Therapy Resistance Mediated by Dynamic Regulation of Extrachromosomal Mutant EGFR DNA

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Materials and Methods

Antibodies and Reagents

Antibodies used for immunocytochemistry, immunohistochemistry and/or western blot included: EGFRvIII (Dako), EGFR (Millipore), p-EGFR^{1086} (Invitrogen), p-S6$^{235/236}$ (Cell Signaling), p-AKT^{473} (Cell Signaling), p-ERK$^{202/204}$ (Cell Signaling), ZO-1 (Cell Singaling), ZEB-1 (Cell Signaling), N-Cadherin (Cell Signaling), Vimentin (Cell Signaling), B-Catenin (Cell Signaling), Actin (Sigma) and Ki67 (Dako).

Cell Culture Conditions

Cell culture consisted of neurosphere culturing conditions consisting of DMEM/F12 (Gibco), B27 (Invitrogen), Glutamax (Invitrogen) supplemented with Heparin (Sigma), EGF (20ng/mL, Sigma) and FGF (20ng/mL, Sigma) added every 3 days. Generation of erlotinib resistant (ER) neurospheres consisted of continuous erlotinib treatment (GBM39: 5μM, 60 days; HK296 and GBM6: 1μM, 30 days) with fresh erlotinib added every 7 days. Assessment of erlotinib resistance was determined through comparing sensitivity with vehicle control (DMSO) by WST-1 Assay (Promega) and Annexin V/PI staining for apoptosis induction (BD Biosciences).

Microfluidic Chip Fabrication

The microfluidic cell array chip was fabricated as previously described (*1*). Briefly, the microfluidic chip for single cell characterization consists of 12-24 individual channels at a width of 50μm and 8mm long at a height of 120 μm. This design was incorporated on a silicon wafer through soft lithography methods. Chip fabrication consisted of adding polydimethylsilloxane (PDMS), a semipermeable polymer, to the wafer, where it was subsequently allowed to cure at 80C overnight. The chip was then removed from the mold where holes, which matched the circumference of a standard 20uL pipette tip, were punched to form the inlet and outlet of each individual channel. Finally, the PDMS chip was plasma treated where it was then attached to a cover slip that was prewashed with

1M HCl overnight at 37°C. The assembled chip was then baked overnight at 80C and coated with 100ug/mL poly-D-lysine (Millipore) in PBS overnight at 4°C.

Phase II lapatinib clinical trial

Details of the phase II lapatinib trial have been described previously (*2, 3*). Briefly, the North American Brain Tumor Consortium (NABTC) trial 04-01 titled "A biomarker and Phase II study of GW572016 (lapatinib) in recurrent malignant glioma" enrolled consented patients from University of California at Los Angeles (UCLA), University of California at San Francisco, Dana- Farber Cancer Center, Memorial Sloan Kettering Cancer Center, University of Pittsburgh, Neuro-oncology branch of National Institutes of Health, University of Wisconsin, and Duke University. Adult patients who had a Karnofsky performance score (KPS) equal to or greater than 60 and were candidates for surgical resection were eligible for this study. Patients were administered 750 mg of lapatinib orally twice a day (BID) for 7 to10 days (depending on whether treatment interval fell over a weekend) before surgery, the time to steady state. Blood and tissue samples were obtained at the time of resection. After recovery from surgery, patients resumed lapatinib treatment at the neoadjuvant dose of 750 mg BID until clinical or radiographic evidence for tumor progression was found. Patients with pre and post lapatinib samples available, scored positive for EGFRvIII and PTEN at initial biopsy (as determined by immunohistochemistry), and demonstrated a decrease in phospho-EGFR staining (to demonstrate pharmacodynamics) were included for this study (n=6). Patients 1, 2, 3, 4, 5 and 6 correspond to E50153, E50038, E50034, E09355, E09333, and E50031 respectively in Vivanco et. al. Intratumoral lapatinib levels for each patient were reported Vivanco et. al.

Human Tumor and GBM Xenograft Specimens

The serially-propagated GBM39 xenograft has been previously described (*4*). Briefly, surgically resected tissue from a GBM patient at the Mayo Clinic, Rochester, MN was mechanically digested and temporarily cultured in vitro for lentiviral infection of firefly luciferase (ff-LUC). Transduced cells were then collected and mixed 1:1 with matrigel and injected subcutaneously in immunocompromised mice. Once tumors were

established, samples were either archived or propagated in vivo through digestion and re-injection. For low passage patient-derived GBM neurospheres, all patients were consented with approved Institutional Review Board from UCLA. Human brain tumor specimens and mouse xenografts were digested into single cell suspensions through enzymatic digestion methods. Specifically, 12500 U of Collagenase II (Worthington Biochemical Corp.) and 12500 U of Collagenase IV (Worthington Biochemical Corp.) were prepared in serum-free Dulbecco's Modified Eagle's medium (DMEM, Gibco). Subsequently, minced tumor samples was added to the mixture and shaken for three hours at 140 RPM at 37° C. The digested samples were then passed through a 40μm filter and centrifuged at 400g for 5 min. Purified pelleted cells were resuspended in serum-free DMEM/F12 (Invitrogen) and loaded on chip or placed in neurosphere culturing conditions containing DMEM/F12, B27 (Invitrogen), Glutamax (Invitrogen) supplemented with EGF (Sigma), FGF (Sigma) and Heparin (Sigma).

Microfluidic Chip Immunocytochemistry

All samples were prepared at a concentration of $1.0x10^6$ cells/mL. Three microliters of the cell suspensions were then loaded onto the poly-D lysine coated microfluidic chip through a standard 20μL pipette. The chip was placed at 37° C for 5 min to allow the cells to adhere inside the microfluidic chip. For fixation, 4% paraformaldyde (Sigma) was added to the channels of the chip containing the adherent cells and allowed to fix 15 min. Following three PBS washes, cells were subsequently permeabilized with .3% Triton X-100 (Sigma) for 10 minutes, followed by three additional PBS washes. Cells were then blocked in 10% normal goat serum for 30 minutes. Antibodies used for immunocytochemistry include: EGFRvIII (Dako), EGFR (Millipore), p-EGFR^{1086} (Invitrogen), p-S6$^{235/236}$ (Cell Signaling), p-AKT^{473} (Cell Signaling) and Ki67 (Dako). To achieve same species antibody multiplexing, all antibodies were primary labeled using Zenon Immunolabeling Technology (Invitrogen). Desired cocktails of various antibodies were diluted in 10% normal goat serum and incubated in the microfluidic chip for 1 hour. Following three PBS washes, cells were counterstained with DAPI (Invitrogen). For TUNEL immunoflourescence staining, prior to primary antibody incubation, cells within the microfluidic channels were analyzed for apoptosis using the In Situ Cell Death

Detection Kit, TMR red, following the manufacturer's protocol (Roche). Cellular analysis was done with Metamorph Software (Molecular Devices, ver 7.5.6.0) using the Multi-Wavelength Cell Scoring module. For each analysis, at least 1000 cells were quantified.

Giemsa-Banded (G-band) Metaphase analyses and Fluorescence in situ Hybridization (FISH)

GBM39 naïve, erlotinib resistant (ER), and erlotinib resistant drug removed (DR) neurospheres grown in neurosphere culturing conditions were subcultured in in-situ dishes for 24-48 hours before using routine cytogenetics procedures of harvesting, fixation and obtaining metapahse preparations. Chromosomal analyses were performed by G-banding using trypsin and Giemsa (GTG) at approximately 400 - 450 band level. Twenty well-spread metaphases from each of the three cell-lines were analyzed to identify the extrachromosomal elements and other marker chromosomes. Fluorescence in situ hybridization (FISH) was performed using commercially available fluorescently labeled dual-color *EGFR* (red)/CEP 7(green) and *MDM2* (red)/CEP 12 (green) probe sets form Abbott-Molecular, IL. USA. The FISH hybridization and analyses were performed on both metaphase an interphase preparations from the three cell-lines and formalin fixed paraffin tissue sections, following the manufacturer's suggested protocols. The cells were counterstained with DAPI and the red and green fluorescent probe signals were simultaneously observed and imaged under a Zeiss (Axiophot) Fluorescent Microscope equipped with dual- and triple-color filters.

^{18}F-Fluorodeoxyglucose (FDG) uptake assay

For *in vitro* ^{18}F-FDG uptake assays, freshly sorted EGFRvIII^{High} and EGFRvIII^{Low} cells GBM39 neurospheres were loaded into one of four microchannels within a microfluidic chip, which was similar to that reported previously (*5, 6*). Each microchannel contained four separate microchambers. Upon cell loading, each of the four microchambers contains between 20 and 61 cells, the numbers of which are counted by microscopic imaging through the transparent elastomer material of the microfluidic chip. The bottom surfaces of the microchambers are coated with fibronectin to support cell attachment, and

are separated by 50 micrometers from a B-camera. This camera uses a position sensitive avalanche photodiode to detect charged particles. After sorting, a cell suspension in cell culture medium with a concentration of 3×10^6 cells/ml was pipetted into the microchannel inlets. The microchannels were gently flushed three times with flowing PBS solution to remove unattached cells and to thoroughly deplete residual glucose in the microchannels. ^{18}F-FDG (radioactivity 1mCi/mL) in PBS was then loaded into the cell chambers by flowing for 30 sec to completely replace PBS. The whole microchip placed in a CO_2 incubator at 37 °C for 30 min, before washing by PBS two times for 30 sec. The chip was then imaged with the B-camera. The radioactivity in each microchamber was normalized against the number of cells in that microchamber for the B-camera image The data of Fig. 1E represents the average of two separate microchip experiments, each one of which yielded between 4 and 8 separate assays for each phenotype.

Xenograft Model

In vivo subcutaneous serial passaging of GBM39 was described previously (*4*, *7*). For in vivo erlotinib (Chemietek) experiments, single-cell suspensions of digested GBM39 xenografts were prepared at a concentration of 1×10^6 cells/ml in a solution of DMEM/F12 containing no additives and Matrigel (BD Biosciences). Two-hundred microliters of this solution was injected into the flanks of NOD-scid IL2Rgamma^{null} (NSG, Jackson Laboratories) mice and then randomly grouped into either vehicle/placebo (n=4), short-term erlotinib (n=4), or continuous erlotinib treatment (n=4). Tumor growth was monitored with calipers measuring the perpendicular diameter of each tumor. Erlotinib was administered daily via oral gavage at a dose of 150mg/kg, which has previously shown to be elicit a response in GBM39 with low toxicity (*4*). All experiments were conducted after approval by the Chancellor's Animal Research Committee of UCLA.

FACS and Flow Cytometry

Single cell suspensions from tumor xenografts were obtained and were incubated with a mixture of antibodies including anti-mouse CD45-APC (BD), anti-mouse H2kd-APC (eBioscience) for mouse cell exclusion and EGFRvIII-Alexa 488 in 5% FBS PBS for 20

min on ice. In addition, propidium iodide was used for dead cell exclusion. For cell sorting, the Aria II flow cytometer was used (BD Biosciences). Acquisitions were performed on a BD LSRII. All data was analyzed using FlowJo (Tree Star) software.

Western Blotting

Western blotting was conducted as described previously (*2*). Cells or tissue samples were homogenized and lysed in RIPA Lysis Buffer containing Tris (10 mmol/liter; pH 7.4), NaCl (100 mM), EDTA (1 mM), EGTA (1 mM), NaF (1 mM), Na4P2O7 (20 mM),Na3VO4 (2mM), 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, leupeptin (10 μg/ml), aprotinin (60 μg/ml), and phenylmethanesulfonyl fluoride (1 mM). Equal amounts of protein extracts were separated by 8 or 10% SDS–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membrane was blocked for 1 hour in tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk and then probed with desired primary antibodies overnight at 4°C, followed by secondary antibodies conjugated to horseradish peroxidase (HRP). The immunoreactivity was revealed by use of an ECL kit (Amersham Biosciences).

Immunohistochemistry

Paraffin-embedded tissue blocks were sectioned using the UCLA Pathology
Histology and Tissue Core Facility. Immunohistochemical staining (IHC) and scoring was performed as previously described (*2, 8-10*). Slides were counterstained with hematoxylin to visualize nuclei. Paraffin-embedded tissue sections underwent immunohistochemical analysis and quantitative image analysis using Soft Imaging System software was performed. We have previously demonstrated the utility of this quantitative method for measuring drug-specific effects in paraffin-embedded tissue samples from GBM patients enrolled in clinical trials with targeted agents (*2, 8-10*).

Cell Death and Proliferation Assay

Relative cell proliferation was determined using the WST-1 Cell Proliferation Assay Kit and absorbance was measured with a microplate reader (Bio-Rad) at 420 to 480. Cell death was determined by trypan blue exclusion assay.

Mapping *EGFRvIII* deletions in glioblastoma extrachromosomal DNA

Extrachromosomal DNA was isolated from glioblastoma neurospheres by a modified Hirt procedure (*11*) and was digested with BamH1 and probed by Southern Blotting with an exon 8 probe to determine the size of the cell-type specific *EGFRvIII* fragments generated by BamH1. Given that there are 14 BamH1 sites in intron 1, one site in intron 6 and one in intron 8 just downstream on exon 8, we reasoned that the *EGFRvIII* deletions could be mapped by sequencing PCR products obtained by combining an intron 8 anchor primer adjacent to the BamH1 site with forward primers that anneal adjacent to each of the 14 BamH1 sites in intron 1. The chromosome 7 coordinates for BamH1 site just upstream on exon 1 was 55070485. Those of 14 BamH1 sites in intron 1 were 55105839, 55107896, 55117201, 55119776, 55120751, 55124472, 55132138, 55146472, 55151633, 55178383, 55187313, 55192289, 55192343 and 55198500. The BamH1 site at intron 6 was at 55220852 and the BamH1 site just downstream on exon 8 was located at 55224167. Only those primer sets yielding a product of similar size to the bands obtained by Southern blot were sequenced. Forward primers (Fig. 3) were:

1: CTATTGGACACAACTGGAAGAAG (Chromosome 7 coordinates, 55070463-55070485),

2: TGGAGCCACAGTACATTCAGGTG (55105817-55105839),

3: GCTCTGTGGAGGTCAGAAGGAG(55107875-55107896),

4:ACATCTCTGGAGAAGGAAATGG(55117180-55117201),

5:GGTGGAGGATGAGAGGACCCTG(55119755-55119776),

6:TAGCACCCTTCAAATGTTTTAG(55120730-55120751),

7:AAGATCTGGGTGTTTTCATTGG(55124452-55124473),

8:AGTATCCAGACATCCAGAAAGG(55132118-55132139),

9:GAGATGGTTTGGGCAGCCCGCG(55146451-55146472),
10:ACATATAGGTTAGCAAGATTGG(55151613-55151634),
11:TTCCATTCTGTTTCCTGTTCGG(55178363-55178384),
12:GTTCAAAAAAAGGTATCCCAAGG(55187292-55187314),
13:GGGCATAACTGATCCAAAGGATG(55192267-55192289),
14:CCAGCCAAGATGACAACAGTCAG(55192321-55192343),
15:TCAGCTGTATGTGCCCCGCACAG(55198478-55198500),
16:CTGGGTTAGGGCCTCCTGACACG (55220830-55220852).

The anchor primer was designed at the BamH1 site in intron 8 (primer 17): GGATTAAGAATAGCTAGGGATCC (55224184-55224162). PCR products of the expected size were obtained from GBM6, GBM39 and HK296 by combining the anchor primer and forward primers 10, 13 and 5, respectively using platinum Taq DNA Polymerase High Fidelity (Life Technologies, Carlsbad, CA). PCR products were cloned using the TOPO TA Cloning Kit (Life Technologies), sequenced and aligned to the *EGFR* gene using the UCSC Genome Browser (Feb 2009 GRCH/hg19 Assembly). Primer pairs 13/17 and 14/17 span regions that are 32kb apart in wild type *EGFR*, but only slightly over 4kb in *EGFRvIII*, thus amplifying *EGFRvIII*, but not wild type *EGFR*. Primers 15 and 16 are both deleted in *EGFRvIII*, but maintained in wild type *EGFR*.

Quantitative PCR analysis of *EGFRvIII* extrachromosomal DNA

Glioblastoma neurosphere cells, $0.5x10^6$ to $1.0x10^6$ cells, were pelleted; lysed and extrachromosomal DNA was isolated as above. Triplicate qPCR reactions containing 1 ng of DNA were run on a CFX96^{TM} Real Time System (Bio-Rad, Hercules, CA) with the following reaction conditions: 95ºC for 5 min, 40 cycles of 95ºC for 15s and 60ºC for 30s. Primer pairs for glioblastoma cell-specific *EGFRvIII* mutants were designed to span the *EGFR* deleted regions. The data was normalized to mitochondrial DNA and the relative amount of *EGFR* extrachromosomal DNA was determined using $2^{-\Delta}$Ct formula. Primers for mitochondrial DNA were, forward: CTCAAATGGGCCTGTCCTTG and reverse: GCTTTGGGTGCTAATGGTGG. Primers for glioma cell-specific EGFR mutants were, GBM39: GGGTCTCATTAGGAAGGAGAGG and

TCTGTGAACCATCTGCTCTTGG, HK296: CTTTGATGTCCCTGCCTGAG and CTGTGTCATCACTGTGGCAC, GBM6: TGTGTAGCGGGTACCTTCTG and TGTGGCTGCTGGATGAGTG.

MDM2 PCR

To detect the presence of MDM2 amplification, five nanograms of Hirt DNA from GBM39 naive, erlotinib resistant (ER) and drug removed (DR) cells was PCR amplified with MDM2 primers (forward: CCCAGGTTAAGAACTTCTGCACTAG reverse: AGGCATAGTGGTACAAGCCTGTAG) mapping to a region of intron 1 that yielded a 960 bp product. One hundred nanograms of fibroblast genomic DNA was used as positive control.

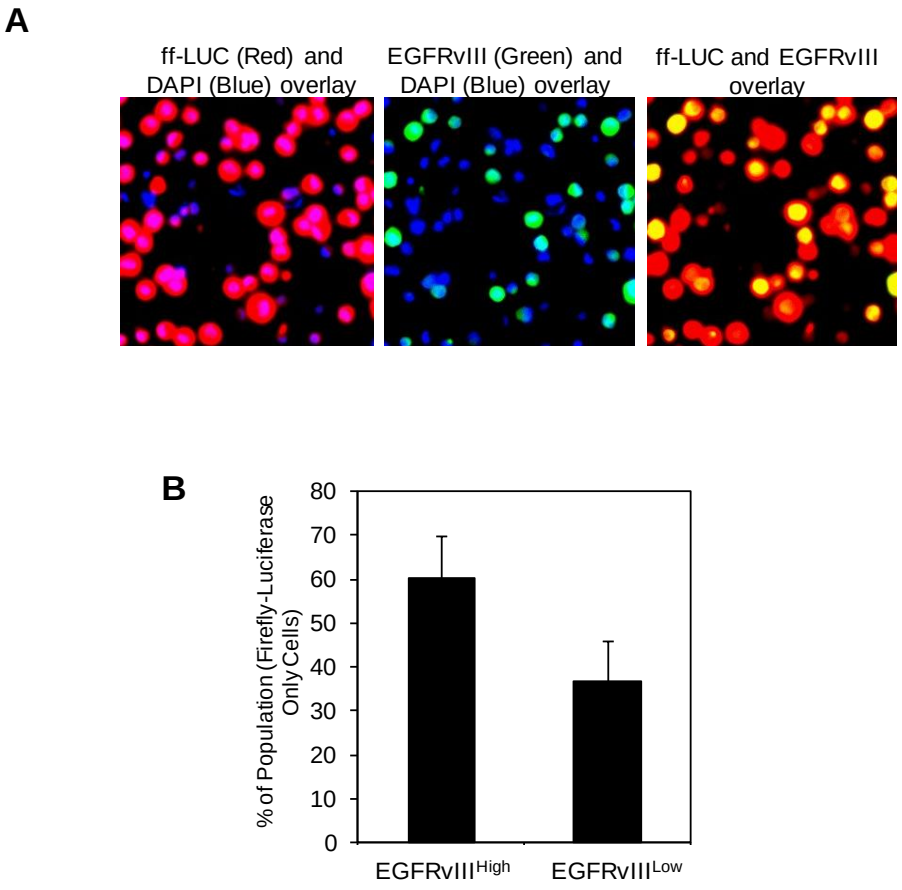

Fig. S1.

Firefly-luciferase labeling identifies tumor cells, enabling assessment of tumor specific EGFRvIII staining (**A**) Co-immunofluorescence of Firefly-luciferase (f-LUC, Red) and EGFRvIII (Green) on MIC chip of GBM39 naïve single cells shows tumor populations containing $EGFRvIII^{High}$ and $EGFRvIII^{Low}$ cells. (**B**) Quantification from MIC staining of $EGFRvIII^{High}$ and $EGFRvIII^{Low}$ cells within the Firefly-luciferase positive population. Quantification based on analysis of >1000 cells. Values are mean ± SEM.

	EGFRvIII^{High}	EGFRvIII^{Low}
Functional Assays		
% Ki67+	20(±4)*	5(±1)
% TUNEL+	.6 (±.1)*	10.4(±0.4)
Signaling Assays		
pEGFR	4140(±230)**	170(±105)
pS6	2930(±400)*	1106(±189)
pAkt	1150(±40)*	460(±60)

Fig. S2

MIC Chip assay quantification of signaling pathways in EGFRvIII^{High} and EGFRvIII^{Low} subpopulations Top: MIC characterization of % positive for Ki67 and TUNEL for the EGFRvIII^{High} and EGFRvIII^{Low} subpopulations. * P<0.005 from unpaired t test. Bottom: MIC analysis of signal intensity for phospho-EGFR (Y1086), phospho-AKT (s473), and phospho-S6 (s235/236). The final intensity was determined following background subtraction (*1*) . Statistics are derived from MIC quantification of >1000 cells. * P<0.005, ** P<0.0001 from unpaired t test.

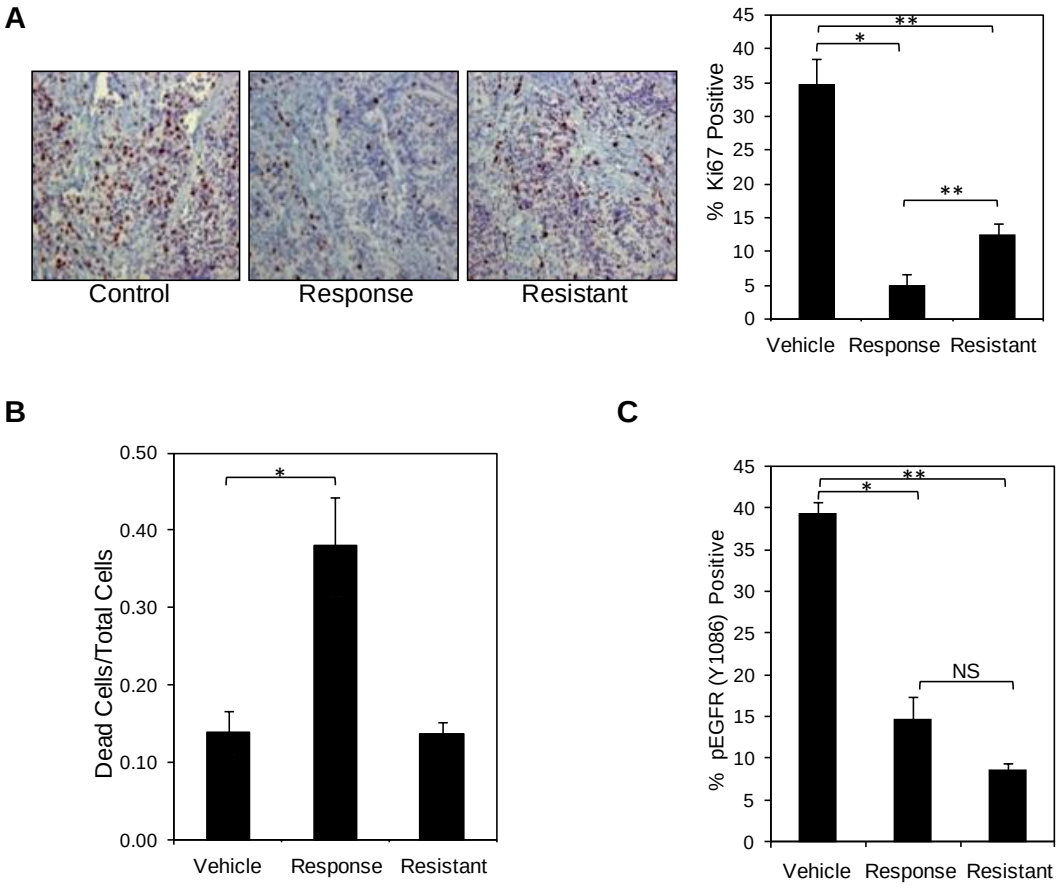

Fig. S3

IHC staining of Ki67, TUNEL and pEGFR in GBM39 xenograft model of acquired erlotinib resistance. (**A**) Ki67 IHC and quantification of vehicle, response and resistant tumor samples. Values are mean± SEM. * $P< 0.001$ ** $P < 0.01$ from unpaired t test. (**B**) Trypan blue exclusion assay on vehicle, response and resistant tumor samples. Values are mean ± SEM. *P < 0.01 from unpaired *t* test. (**C**) MIC analysis of pEGFR (Y1086) intensity in vehicle, response and resistant samples. Values are mean ± SEM. * $P<0.01$ ** $P<0.0001$ from unpaired *t* test.

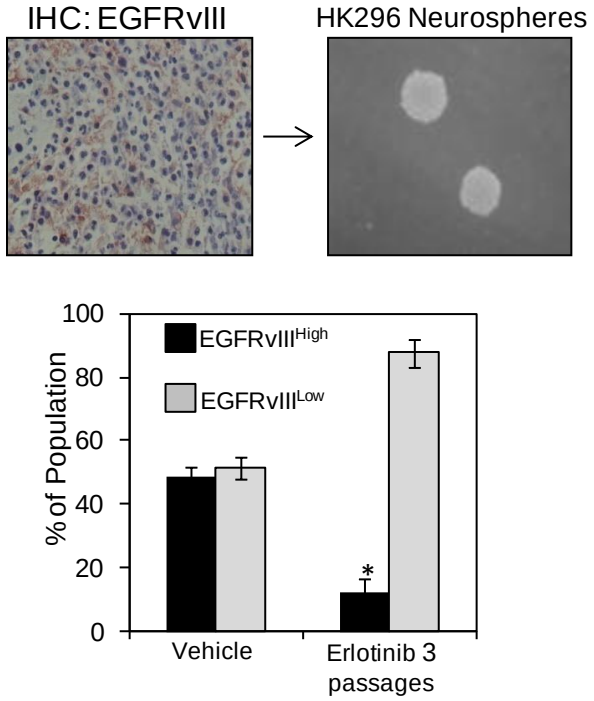

Fig. S4

Effect of erlotinib on EGFRvIII High/Low ratio in an independent patient-derived GBM neurosphere model. *Top:* Heterogeneity of EGFRvIII expression by immunohistochemistry in patient biopsy tissue in which HK296 was derived. *Bottom:* MIC quantification of EGFRvIII in HK296 patient-derived neurospheres shows enrichment for EGFRvIIILow cells following three passages of erlotinib treatment. Cellular composition statistics derived from MIC quantification of >1000 cells. Values are mean ± SEM * $P<0.05$ from unpaired *t* test

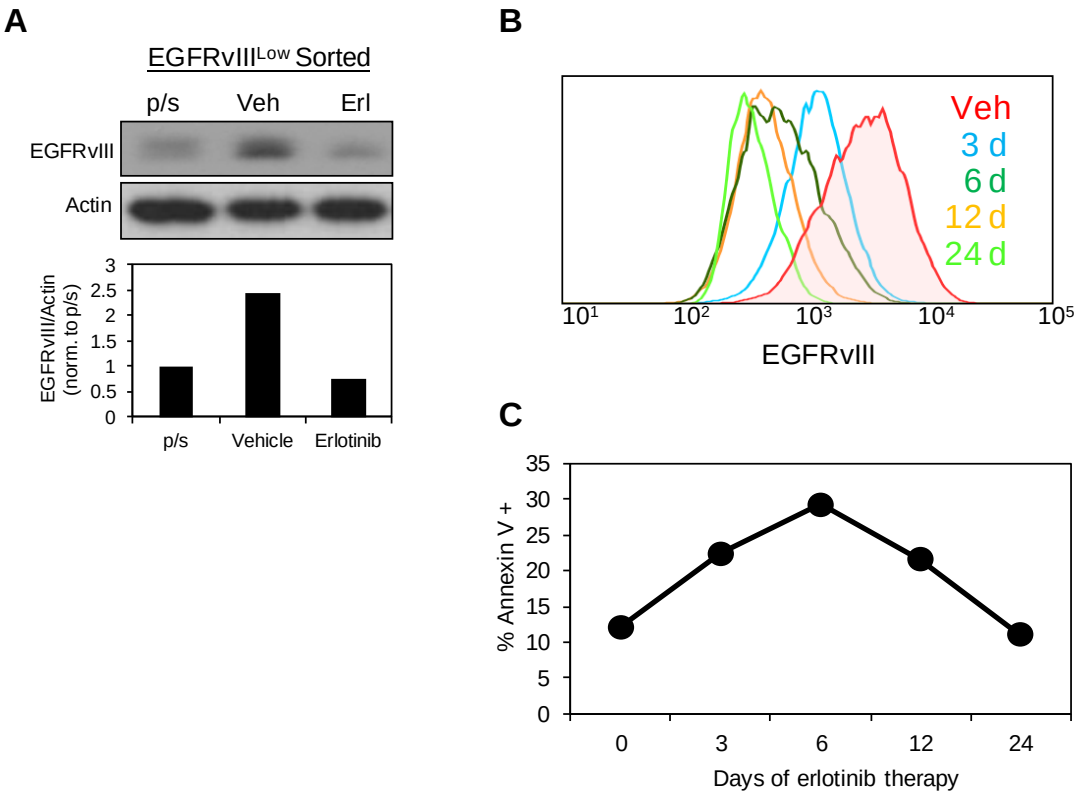

Fig. S5

Erlotinib prevents upregulation of EGFRvIII expression. (**A**) FACS-sorted EGFRvIII^{Low} GBM39 cells were analyzed for EGFRvIII expression by immunoblot analysis immediately post-sort, or after 24 hours with vehicle or erlotinib treatment (5μM). (**B**) FACS analysis of EGFRvIII expression after 3, 6, 12, 24 days of erlotinib or vehicle (Veh) in GBM39 naïve cells. (**C**) Annexin V staining from (B).

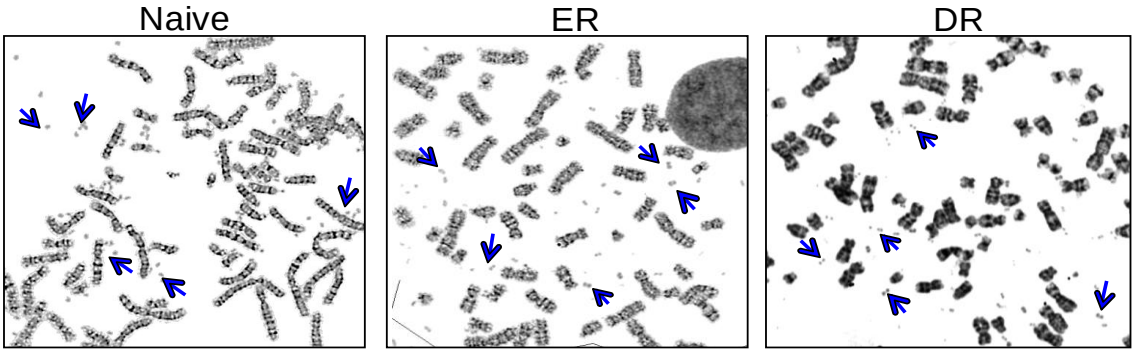

Fig. S6

Geimsa Banding on GBM39 naïve, ER and DR cells. Partial Giemsa banded metaphases from naïve, erlotinib resistant (ER) and drug removed (DR) cells showing abundant extrachromosomal DNA in all conditions (blue arrows).

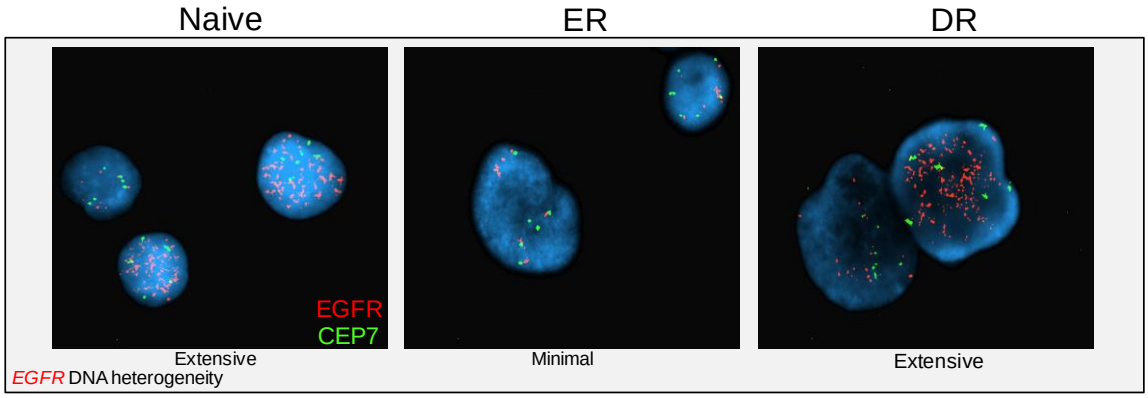

Fig. S7

***EGFR* FISH on interphase cells of GBM39 cells.** Representative images from dual-color FISH (*EGFR* (red)/CEP7 (green)) on GBM39 naïve, erlotinib resistant (ER) and drug removed cells (DR). Nuclei counterstained with DAPI.

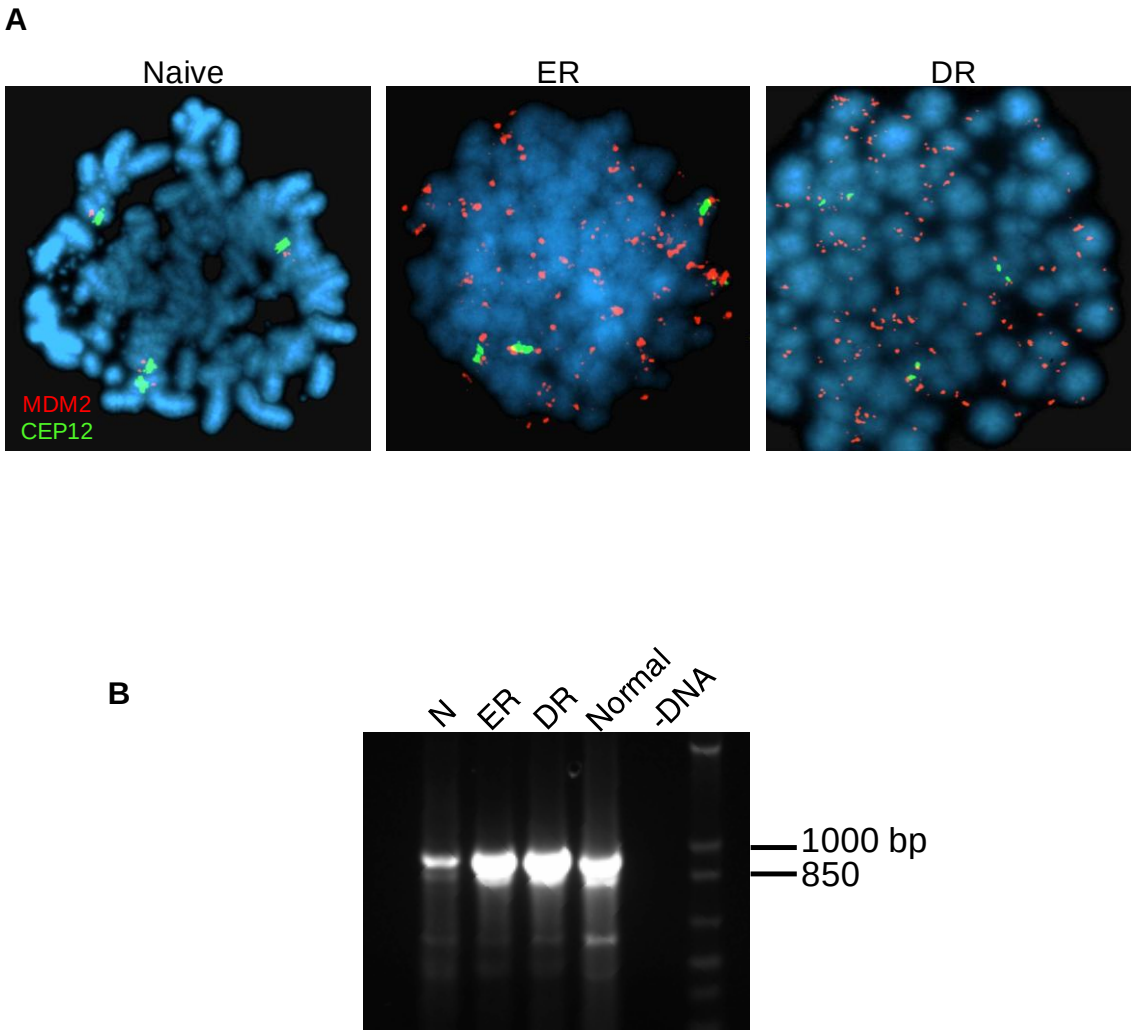

Fig. S8

Analysis of MDM2 in erlotinib resistant GBM cells. (**A**) FISH analysis of a metaphase spread using *MDM2* (red) and chromosome 12 centromere probes (green) reveals abundant *MDM2*+ DMs. (**B**) PCR amplification of a 960 bp region from intron 1 of *MDM2* using 5 ng of low molecular weight DNA isolated from naïve, erlotinib resistant and drug removed GBM39 cells. 100 ng of normal genomic human DNA is used as a control.

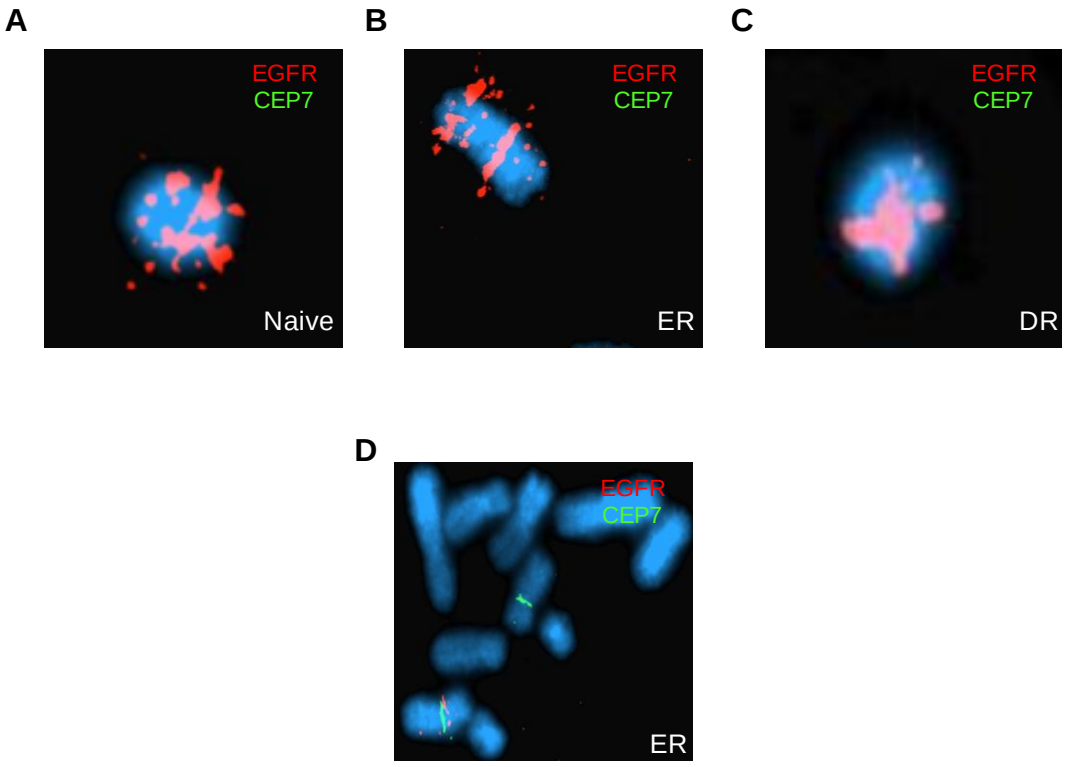

Fig. S9

Metaphase FISH of DAPI stained chromosomes using *EGFR* (red) and chromosome 7 (green) probes reveals "marker" chromosomes characterized by many copies of *EGFR*, but no chromosome 7 centromere. (**A**) Treatment naïve, (**B**) erlotinib resistant (ER) and (**C**) erlotinib resistant drug removed (DR) cells. (**D**) Another region as a positive control for CEP7 FISH probe in ER cells.

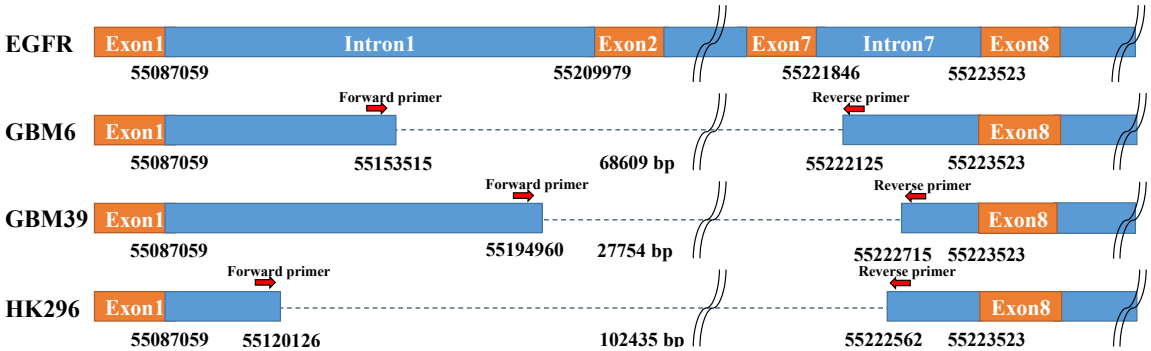

Fig. S10

Primer design for qPCR of extrachromosomal *EGFRvIII* in GBM6, GBM39 and HK296. Primer sequences were designed to the unique breakpoints adjacent to exons 2-7 within *EGFR* for GBM6, GBM39, and HK296 specific for *EGFRvIII*.

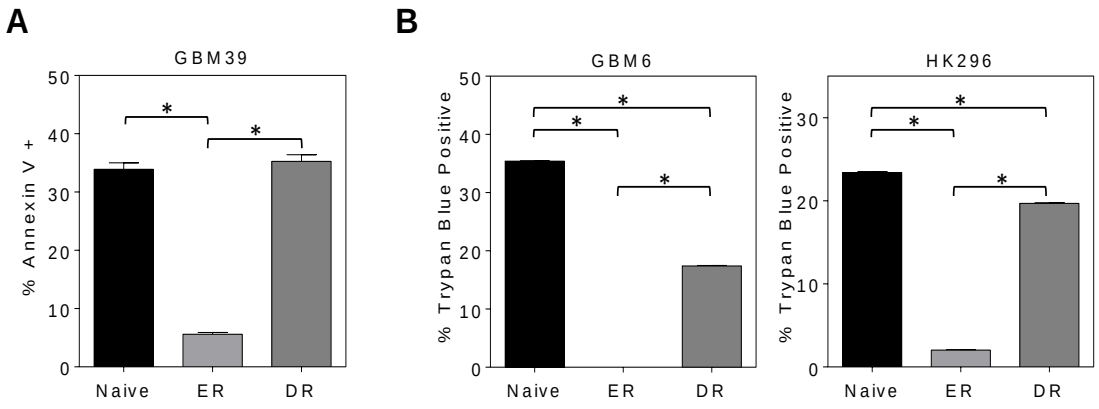

Fig. S11

GBM patient-derived neurospheres are re-sensitized to erlotinib following drug removal of erlotinib resistant cultures. (**A**) Annexin V staining of GBM39 treatment naïve, erlotinib resistant (ER) and erlotinib resistant drug removed (DR, 30 days) cells following 72 hrs of erlotinib treatment (5μM). Values are mean ± SEM. * $P< 0.005$ from unpaired *t* test. (**B**) Trypan blue exclusion assay of GBM6 and HK296 treatment naïve, erlotinib resistant (ER) and erlotinib resistant drug removed (DR, 72 hrs) cells following 72 hrs of erlotinib treatment (GBM39: 5μM, GBM6 and HK296: 1μM). Values are mean ± SEM. * $P<0.0001$ from unpaired *t* test.

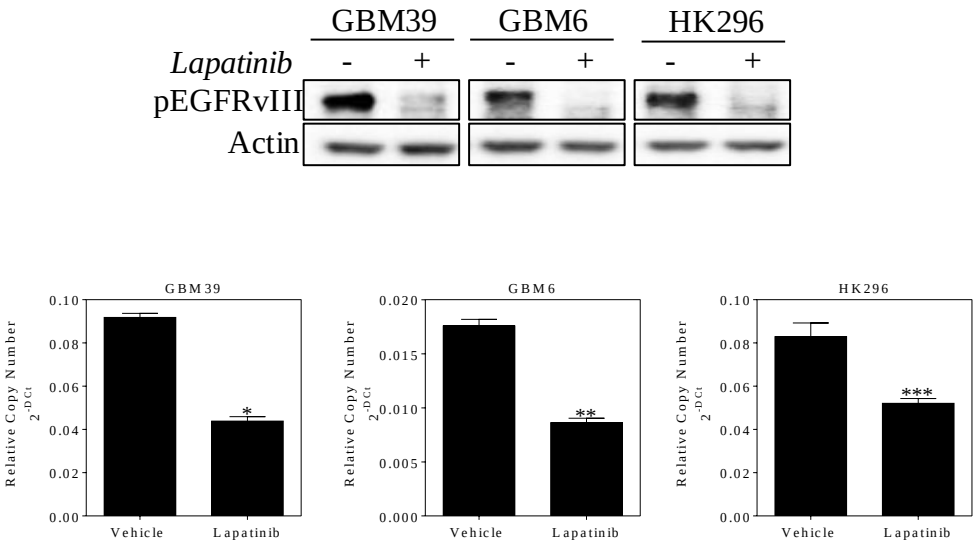

Fig. S12

Lapatinib reduces *EGFRvIII* extrachromosomal levels in GBM39, GBM6 and HK296 neurospheres. (Top) Immunoblot of GBM39, GBM6 and HK296 following 48 hrs treatment with 1.5μM lapatinib. (Bottom) qPCR analysis of extrachromosomal *EGFRvIII* levels in GBM39, GBM6, and HK296 cells following treatment with 0.5 μM lapatinib for 7 days followed by 1.5μM for 7 days. **P* <0.0001, ** *P* <0.001, *** *P* <0.01.

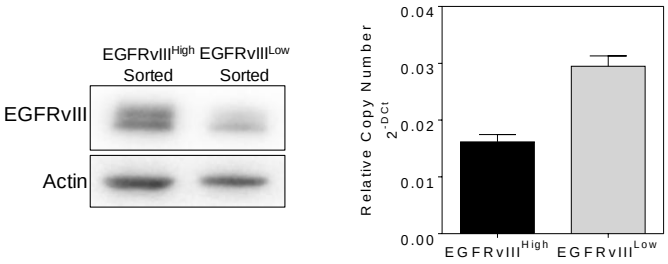

Fig. S13

EGFRvIII extrachromosomal levels in FACS sorted EGFRvIII^{High} and EGFRvIII^{Low} cells from GBM39 naïve tumor cells. GBM39 naïve neurospheres were FACS sorted for EGFRvIII^{High} and EGFRvIII^{Low} subpopulations. Sorted populations were analyzed for EGFRvIII protein (left) and *EGFRvIII* extrachromosomal levels by qPCR (right).

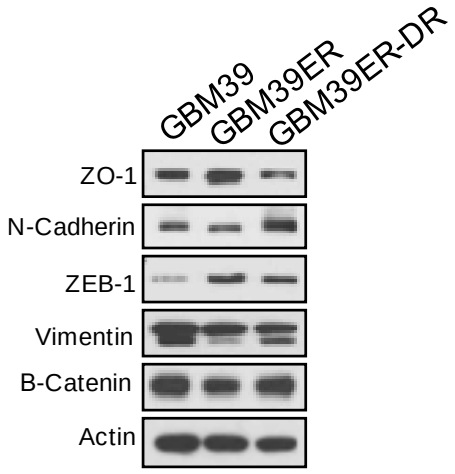

Fig. S14

Irreversible upregulation of ZEB-1 in GBM39 erlotinib resistant (ER) and drug removed (DR) cells. Immunoblot of GBM39 naïve, erlotinib resistant (ER) and drug removed (DR) for markers of epithelial–mesenchymal transition**.**

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